

METAL ION INHIBITION OF CORN ROOT PLASMA MEMBRANE ATPASE

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Abstract—In the presence of K^+ , the hydrolysis of ATP catalysed by the ATPase of corn plasma membrane showed negative cooperative kinetics. When the complexes of ATP and Mg^{2+} , Mn^{2+} , Ca^{2+} or Cd^{2+} were used as substrates, the catalysed hydrolysis changed to follow simple Michaelis–Menten kinetics. However, this change was not observed with Zn^{2+} –ATP as the substrate. A substantial enhancement of the hydrolysis was observed only when the complexes of Mg^{2+} and Mn^{2+} were used. Kinetic parameter determination indicated that the enzyme exhibited a similar binding property but a different catalytic efficiency to Mg^{2+} , Mn^{2+} and Ca^{2+} –ATP. The enzyme formed a more stable but less reactive complex with Cd^{2+} –ATP. The presence of aluminium ions competitively inhibited the membrane-catalysed hydrolysis of Mg^{2+} –ATP, but showed no effect when free ATP was the substrate. This finding suggested that aluminium might bind in the vicinity of the Mg^{2+} of Mg^{2+} –ATP in the active site of the enzyme. On the basis of these observed inhibitory effects, possible origins of metal ion toxicity to root plasma membrane ATPase activity are discussed.

INTRODUCTION

The long-term effects of certain toxic metal ions, e.g. Al^{3+} , Mn^{2+} and Cd^{2+} , to plants are well documented [1, 2]. However, relatively little information on the molecular origins of the toxicity is available. It has been reported that Al^{3+} may form complexes with nucleic acid [3]; interfere with protein synthesis [4]; change artificial membrane fluidity [5]; and alter the uptake of essential elements [6]. The presence of Mn^{2+} may increase the activity of auxin oxidase [7]; enhance catalase, peroxidase and polyphenol oxidase activities [8]; inhibit nitrate reductase activity [9]; and decrease the concentration of Ca^{2+} in plants [7]. The toxic effects of Cd^{2+} may be mostly attributed to its ability to interact with sulphhydryl and imidazole groups of enzymes [10] and phosphatidylethanolamine and phosphatidylserine of biomembranes [11]. Despite this information, there has been very little systematic investigation on the possible toxic effects of these metal ions to the plant root plasma membrane, which is the first true diffusion barrier between root cells and the surrounding soil.

The plasma membrane of plant root cells contains an ATPase system which utilizes Mg^{2+} –ATP as its preferred substrate [12–15]. The hydrolysis of ATP catalysed by this membrane enzyme follows a simple Michaelis–Menten scheme [16–18]. In many regards, the properties of this enzyme are similar to those of fungal plasma membrane H^+ –ATPase [15]. Indeed, both the fungal [19, 20] and the plant root [18, 21–23] plasma membrane ATPase contain a 100 k polypeptide which forms a phosphorylated intermediate in the process of catalysing the hydrolysis of ATP. Furthermore, the highly purified fungal plasma membrane ATPase [24] and the partially purified root plasma membrane ATPase [25]

have been reconstituted into phospholipid vesicles. The reconstituted systems exhibit an ATP hydrolysis-supported electrogenic proton pumping activity. Thus, it is likely that plant root plasma membrane ATPase may play a central role in the transport processes occurring between the soil–root interface [12].

Unlike the F_0F_1 -type of ATPases found in mitochondria, chloroplasts and bacteria, the fungal and plant root plasma membrane ATPase are insensitive to the presence of oligomycin [16, 26]. These plasma membrane ATPases share striking similarities with E_1E_2 -type ion translocating ATPases found in animal cells [15, 27]. In a previous report [28], we demonstrated that the hydrolysis rate of ATP catalysed by corn root plasma membrane can be decreased by the presence of ADP, a product of the reaction. Furthermore, the presence of ADP also causes the root plasma membrane ATPase to be less sensitive to the inhibition induced by a wide range of covalent and non-covalent modifiers. These results lead us to propose that in addition to exhibiting product inhibition, the binding of ADP to the membrane also stabilizes the conformation of the active site of the ATPase.

In the present report, we studied the substrate preference of root plasma membrane ATPase by replacing Mg^{2+} with Ca^{2+} , Mn^{2+} , Cd^{2+} or Zn^{2+} . In addition, we also investigated the effects of aluminium ions on the activity of the enzyme. The basis for kinetic effects of metal ion replacement and a probable molecular origin of metal ion toxicity to the ATPase were postulated from the obtained data.

RESULTS

Effects of divalent cations on the enzymatic activity

It has been shown that the hydrolysis of ATP catalysed by corn root plasma membrane can be affected by divalent

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cations [15]. We observed that the enzymatic activity measured in the presence of 3.7 mM ATP in the basal solution was normally 12 $\mu\text{mol Pi}$ released per mg of total membrane protein per hr. The addition of 3.7 mM Mg^{2+} , Mn^{2+} , Ca^{2+} , Cd^{2+} or Zn^{2+} changed the activity by 2.1, 1.7, 0.9, 0.6 or 0.5-fold, respectively. The observed order of divalent cation preference, i.e. $\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+}$, is consistent with previous findings [29]. It is clear that the hydrolysis of ATP is actually inhibited by the presence of the latter two cations. The presence of Ca^{2+} seems to have little effect on the activity. However, this approach, in which only a single equivalent concentration of metal ion and ATP was used to determine the enzyme activity, yielded no useful information to assign the molecular origin of the observed metal ion effects.

Effects of cation-ATP complexation on the enzyme activity

It is known that divalent metal ions interact with ATP to form 1:1 complexes, and at neutral pH (6.5) and 40° the apparent dissociation constants of Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} complexes are 115, 52.5, 23.4 and 19.5 μM , respectively [30]. Thus, in order to understand the aforementioned effects, it is necessary to take this complex formation into consideration. Experiments which kept the total concentration of ATP constant while changing the concentration of divalent cations were performed (Fig. 1). In the case of Mg^{2+} and Mn^{2+} , the enzyme activity showed a bell-shaped metal ion concentration dependence. The presence of Ca^{2+} -ATP or Cd^{2+} -ATP did not significantly affect the enzyme activity, except at higher concentrations when inhibition was detected. It is also clear from the figure that Zn^{2+} -ATP is a poor substrate for the enzyme.

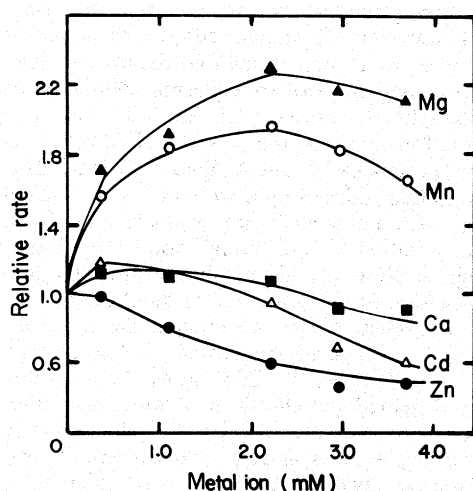


Fig. 1. Inhibition or stimulation of ATPase activity by divalent cations. The relative rate of hydrolysis of 3.5 mM ATP was determined in the presence of various concentrations of Ca^{2+} (■), Cd^{2+} (△), Mg^{2+} (▲), Mn^{2+} (○) or Zn^{2+} (●) in the basal assay medium at 40° . The relative rate of hydrolysis was determined by assigning a value of 1.0 to the rate (11.2 $\mu\text{mol Pi/mg} \cdot \text{hr}$) obtained with no metal present.

Kinetic parameters of the catalysed ATP hydrolysis

The data presented in Fig. 1 indicate that the enzyme can use metal-ATP complexes as the substrate. In addition, the activity depends on the ratio of added divalent cation to ATP. In order to determine the kinetic parameters of the catalysis, experiments were performed in which the ratio of divalent cation to ATP was kept at 1.0 (Fig. 2). As shown in Fig. 2, the non-linear double-reciprocal relationship obtained with ATP alone (not shown) or $\text{ATP} + \text{Zn}^{2+}$ suggests a negative cooperative effect of the hydrolysis process. However, the hydrolysis of ATP changes from a non-linear $1/V$ vs. $1/S$ relationship to a simple Michaelis-Menten dependence in the presence of Mg^{2+} , Mn^{2+} , Ca^{2+} or Cd^{2+} , as shown in Fig. 2 for Mg^{2+} and Mn^{2+} . It is interesting to note that Ca^{2+} -ATP and Cd^{2+} -ATP are less preferred, but can still affect the enzyme to alter its kinetic responses. The K_m and V_{\max} relative to the values obtained with Mg^{2+} -ATP (0.5 mM and 25 $\mu\text{mol Pi/mg} \cdot \text{hr}$), are 1.1 and 0.42, 1.2 and 0.88, and 0.50 and 0.20 for Ca^{2+} -ATP, Mn^{2+} -ATP and Cd^{2+} -ATP, respectively. It is clear from the data that Mg^{2+} -ATP, Mn^{2+} -ATP and Ca^{2+} -ATP have similar binding properties to the active site of the enzyme. But the enzyme catalytic efficiency is different from substrate to substrate. On the other hand, the binding of Cd^{2+} -ATP seems to be able to lock the enzyme in a less reactive conformation. Under the experimental conditions employed, there was essentially no free ATP in the presence of Zn^{2+} . Thus, we conclude that Zn^{2+} -ATP, unlike other metal-ATP complexes, does not interact strongly with the enzyme to alter its kinetic properties.

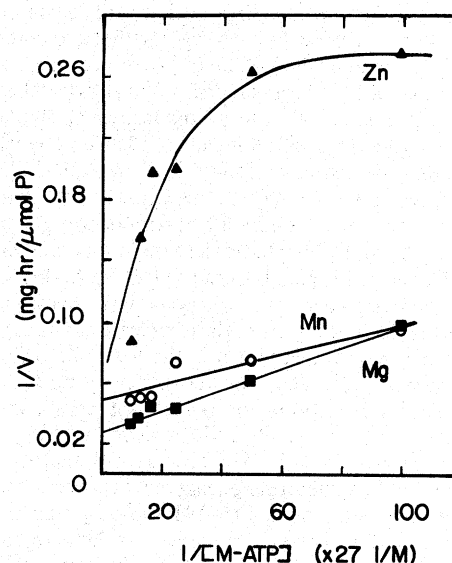


Fig. 2. The kinetics of metal-ATP hydrolysis catalysed by the plasma membrane of corn roots. The Lineweaver-Burk plots of varying metal-ATP concentrations were determined in the basal solution. A linear double-reciprocal relationship was obtained with Mg^{2+} , Mn^{2+} , Ca^{2+} or Cd^{2+} -ATP as the substrate. A non-linear relationship was observed with free ATP or Zn^{2+} -ATP. ■—■, ○—○ and ▲—▲ represent the $1/V$ vs. $1/S$ relationship obtained with Mg^{2+} , Mn^{2+} and Zn^{2+} -ATP, respectively.

Effects of divalent cations on Mg-ATPase activity

Although the plasma membrane ATPase prefers to use Mg^{2+} -ATP as its substrate, it is clear from our data that the presence of other divalent cations will affect the Mg^{2+} -ATPase activity. In order to obtain more quantitative information on the inhibition, the experiments described in Fig. 3 were performed. In these experiments, the concentrations of Mg^{2+} and ATP were kept at 8.5 and 3.5 mM, respectively. The ATP hydrolysis rate was determined in the presence of other divalent cations. As shown, Zn^{2+} which inhibits 50% of Mg^{2+} -ATPase activity at a concentration of about 48 μM ($\text{Zn}^{2+}/\text{Mg}^{2+}$ ratio = 0.0056) is the most effective divalent inhibitor. The same 50% inhibition caused by Cd^{2+} is obtained when the $\text{Cd}^{2+}/\text{Mg}^{2+}$ ratio is 0.015. On the other hand, the inhibition by Mn^{2+} becomes significant (> 20%) only when the $\text{Mn}^{2+}/\text{Mg}^{2+}$ ratio is greater than 0.09. In general, under the experimental conditions, the order of inhibitory efficiency is $\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+} > \text{Mn}^{2+}$ for any given $\text{M}^{2+}/\text{Mg}^{2+}$ ratio. This observed order of inhibition is in agreement with the determined order of substrate preference of the plasma membrane ATPase.

Inhibition of ATP hydrolysis by aluminium ions

We routinely observed that the presence of minimal concentrations of aluminium ions caused a significant decrease in the hydrolysis rate of Mg^{2+} -ATP catalysed by the plasma membrane ATPase. As shown in Fig. 4, this inhibition occurred in the pH range of 5.0–7.0 and the shift of pH dependence of the enzyme activity caused by aluminium ion is similar to that of vanadate. The observed inhibition may be due to a direct binding of aluminium ion to membrane proteins or an indirect consequence of aluminium ion binding to phospholipids in the membrane. However, measurement of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated in the membrane revealed that the polarization (0.30 ± 0.01) was not affected by the presence of aluminium ions (up to

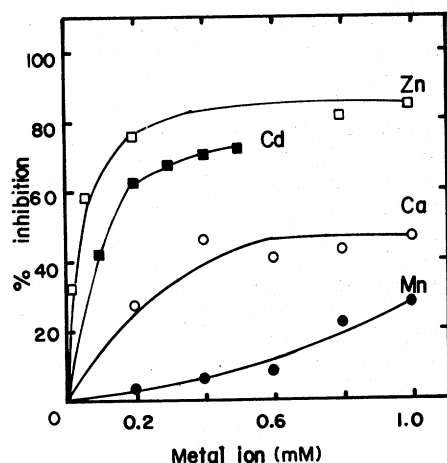


Fig. 3. Inhibition of Mg^{2+} -ATPase activity by divalent cations. The Mg^{2+} -ATP hydrolysis rates were determined in the assay media in the presence of other indicated metal ions. The initial concentrations of ATP and Mg^{2+} were 3.7 and 8.5 mM, respectively. The obtained rate ($28.5 \mu\text{mol Pi/mg} \cdot \text{hr}$), in the presence of Mg^{2+} alone, was taken as 0% inhibition.

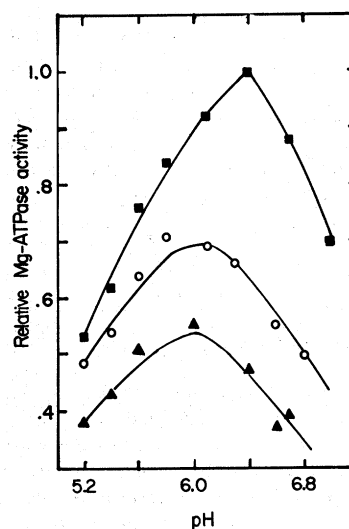


Fig. 4. The pH dependence of Al^{3+} inhibition. The Mg^{2+} -ATPase activity of corn root plasma membrane was measured in the basal assay medium containing 3.7 mM of Mg^{2+} -ATP at different pHs with the specified additives (no additive ■—■, 30 μM aluminium sulphate ○—○, or 0.125 mM vanadate ▲—▲). The relative activity was determined by assigning a value of 1.0 to the highest observed Pi release rate in the absence of any additive ($27.5 \mu\text{mol Pi/mg} \cdot \text{hr}$).

0.02 mM). This result suggests that under the experimental conditions the binding of aluminium ions to the lipid phase of the membrane is insignificant.

Kinetic effects of aluminium inhibition

Due to its limited solubility in the pH range of 6.2–6.6, the total concentration of aluminium species in the assay solution was never greater than 0.2 mM in the present study. The effects of aluminium ionic species on the kinetics of plasma membrane Mg^{2+} -ATPase activity were analysed by a double-reciprocal plot. As shown in Fig. 5A, the presence of 36 μM aluminium ions increases the K_m but has no significant effect on the V_{max} of the enzyme. Thus, aluminium ions apparently behave like a competitive inhibitor. Under the experimental conditions mentioned in Fig. 5A the apparent inhibition constant, K_i , of aluminium ions was determined as 40 μM . A similar competitive pattern was also observed in the inhibition of hexokinase by aluminium ions [31].

Origin of aluminium inhibition

It has been demonstrated from NMR investigations that ATP forms a variety of complexes with aluminium ions over a wide range of pH [32]. From the data given in a paper by Karlik *et al.*, we estimated that at pH 6.6 the dissociation constant of the Al^{3+} -ATP complex is 0.83 mM. As mentioned before, the stability constant of Mg^{2+} -ATP in the same pH range is 50 μM . Thus, it is unlikely that the Al^{3+} -ATP complex functions as a competitive inhibitor to the Mg^{2+} -ATPase activity under the experimental conditions employed in the present study. This conclusion is also supported by the observation that both the competitive inhibition pattern and the value of the apparent K_i are only slightly affected by a

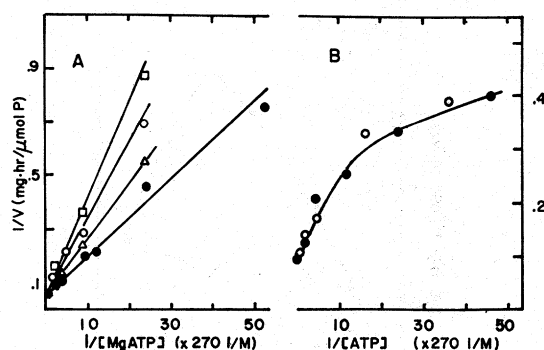


Fig. 5. Effects of aluminium ions on the kinetics of Mg^{2+} -ATP and free ATP hydrolysis. (A) The Lineweaver-Burk plots of the Mg -ATPase activity were determined with and without aluminium sulphate in the basal assay solution at pH 6.6. \bullet — \bullet , Δ — Δ , \circ — \circ and \square — \square represent the $1/V$ vs. $1/S$ plots obtained in the presence of 0, 18, 36 and 76 μM aluminium sulphate, respectively. It was found that the competitive pattern of inhibition prevailed in the presence of various amounts of aluminium sulphate. (B) The same experiments as mentioned in (A) were performed in the absence of Mg^{2+} . The non-Michaelis-Menten kinetic patterns were obtained with (\bullet) and without (\circ) 36 μM Al^{3+} .

wide-range variation of Al^{3+} to Mg^{2+} ratios in the assay medium (data not shown).

However, as shown in Fig. 5B, in the absence of Mg^{2+} neither the kinetic pattern nor the activity of ATP hydrolysis catalysed by the enzyme is affected by the presence of aluminium ions. It seems that the inhibitory effect of aluminium ions becomes evident when the enzyme substrate is in the form of Mg^{2+} -ATP. This result strongly suggests that the location of effective aluminium binding is very close to the active site of the enzyme. It is known that aluminium ion may exist in many different forms and the distribution of different aluminium species shows a complicated pH and concentration dependence [33]. No attempt was made to identify the exact form of aluminium ion that is responsible for the observed inhibition.

DISCUSSION

The involvement of multiple binding of nucleotides to regulate mitochondrial ATPase activity is well documented in the literature [34]. For the E_1E_2 -type of ion translocating ATPases, allosteric modulation of enzyme activity by multiple binding of substrate is also quite common. It was reported, based on kinetic measurements, that the plasma membrane ATPase of *Neurospora crassa* contains two ATP binding sites which are involved alternately in catalysis [35]. Similar allosteric involvement of ATP binding is also suggested to exist in gastric H^+ - K^+ ATPase [36].

Based on the regulatory effects of ADP on corn root plasma membrane ATPase activity, we proposed that the enzyme may contain more than one binding site for ADP [28]. The observation that the inhibition mode to the

ATPase activity caused by DCCD modification showed a substrate concentration dependence also suggests more than one binding site for Mg^{2+} -ATP [37]. In the present study, the apparent negative cooperativity of the ATPase activity observed with free ATP as the substrate also suggests the existence of multiple binding sites for ATP. The linear double-reciprocal relationship obtained with Mg^{2+} -ATP and its similar metal-ATP complexes (Fig. 2) does not necessarily argue against the allosteric regulation of the ATPase activity. It is possible that the transition from a high to a low activity state of the enzyme may be achieved by these substrates at a concentration level much higher than that used in the figure. It should be mentioned that evidence for multiple Mg^{2+} -ATP binding at 16° was observed in the plasma membrane ATPase of barley roots [38]. Whether the enzyme contains more than one binding site in the same polypeptide chain or the enzyme forms an interacting dimer or a higher-order aggregate as a functional entity in the membrane is unknown at the present time. Nevertheless, it seems that the allosteric interaction must be considered to understand fully the reaction mechanism of root plasma membrane ATPase.

While the ATPase of corn roots used Mg^{2+} -ATP as its preferred substrate, the presence of other metal ions may perturb the enzyme activity. The results obtained in the present study provide an assessment on the likely molecular origins of the inhibitory effects of some metal ions. If indeed the enzyme contains non-catalytic substrate binding sites, then the relative binding strength as well as the resulting allosteric effects to the active site may be quite different from substrate to substrate. Quantitative evaluation on this aspect must await the availability of the enzyme in pure form.

In terms of the fate of different substrates at the active site, the inhibition may be grouped into two different categories. For Zn^{2+} , the favourable interaction with ATP leads to the formation of a complex which, like free ATP, does not interact strongly with the enzyme. In the case of Cd^{2+} , the ATP complex formed may be processed in a similar kinetic pathway as that of Mg^{2+} -ATP. However, the catalytic breakdown of Cd^{2+} -ATP is much slower than that of Mg^{2+} -ATP. In either case the inhibition is the result of a change in the property of the substrate. Because of its rather unfavourable interaction with ATP under physiological pH, the aluminium ion presumably exerts its inhibitory effect via a direct interaction with the enzyme. The finding that the inhibition is observed when Mg^{2+} -ATP but not free ATP is used as a substrate strongly suggests that the aluminium ion may interfere with the binding of the Mg^{2+} of Mg^{2+} -ATP in the active site. Thus, the inhibition caused by metal ion to the ATPase activity may be a result of either the formation of inefficient substrates or interaction with the enzyme directly. The overall inhibitory effect is then determined by the binding of substrate to both the non-catalytic and the active sites of the enzyme.

EXPERIMENTAL

Isolation of plasma membrane. Plasma membrane enriched microsomal fraction was isolated from the roots of germinated corn seeds (FRB-73, Illinois Foundation Seeds*) according to a similar procedure of Leonard and Hotchkiss [29] as described in our previous report [37]. The plasma membrane fraction obtained was frozen in liquid N_2 and stored at -20° until use. The protein content was determined by the biuret method.

*Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Hydrolysis of ATP. The hydrolysis of ATP catalysed by plasma membrane at 40° was measured by the appearance of Pi in 1.0 ml of a basal soln at pH 6.2 (unless otherwise specified). The soln contained 30 mM MES, 50 mM KCl, 35 µg membrane protein, 2.8 µg oligomycin and, depending on the specific expt, 5 mM MgCl₂ and/or various concns of an individual cation such as Al [Al₂(SO₄)₃], Mn (MnCl₂), Ca (CaCl₂), Cd (CdCl₂) or Zn (ZnCl₂). The reaction was started by the addition of either ATP or metal-ATP and was terminated by the addition of 1.0 ml of ice-cold 5% trichloroacetic acid. The time intervals selected ensured that < 2% of the ATP was hydrolysed. The amount of Pi formed was determined by the malachite green molybdate complex method as previously described [28]. An aliquot of 50 µl of acid-quenched assay soln was added to 0.5 ml of 0.023% malachite green and 2% molybdate in 2 M H₂SO₄. After incubation of 10 min for colour development at room temp., 2 ml 2 M HCl was added. The absorbance of diluted soln at 660 nm was used for Pi determination. It should be mentioned that the presence of the metal ions used in this study did not affect the determination. The initial rate of ATP hydrolysis was expressed as µmol of Pi released per mg of total membrane protein in 1 hr (µmol Pi/mg · hr).

Purity of the ATPase activity. The ATPase activity of the membrane preparation used in this study was found to be insensitive (< 15% inhibition) to the addition of oligomycin (80 µg per mg of total membrane protein), NaN₃ (5 mM), KNO₃ (100 mM) and ammonium molybdate (0.1 mM) in the basal soln with 5 mM MgCl₂, indicating the presence of a minimal contamination of mitochondrial F₁F₀, free F₁, tonoplast and other non-specific soluble ATPase activities [15, 25]. Among the possible contamination, the tonoplast ATPase is known to be activated by chloride (stayed at an activated plateau between 50 and 100 mM chloride) but inhibited by nitrate (over 90% inhibition at 50 mM nitrate) [39]. Since the total chloride concn in the expt was always in the plateau range and also no nitrate was used at all, the observed activity change caused by the addition of metal salts cannot be attributed to the possible tonoplast contamination.

Measurement of fluorescence polarization. The incorporation of 1,6-diphenyl-1,3,5-hexatriene (DPH) into the plasma membrane was achieved by incubating the plasma membrane (300 µg/ml) in a soln consisting of 50 mM KCl, 30 mM MES (pH 6.3) and 1.90 µM DPH for 10 min at 40°. The sample was illuminated with vertically polarized light (419 nm) and the fluorescence intensities at the parallel (I_{||}) and the perpendicular (I_⊥) directions were recorded at 433 nm. The polarization was calculated by the ratios of (I_{||} - I_⊥)/(I_{||} + I_⊥). The effects of Al³⁺ on the polarization were determined by including various concns (0–0.2 mM) in the incubation medium before the measurement.

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